ADAMTS13 gene deletion enhances plasma high-mobility group box1 elevation and neuroinflammation in brain ischemia–reperfusion injury

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Abstract: Highly adhesive glycoprotein von Willebrand factor (VWF) multimer induces platelet aggregation and leukocyte tethering or extravasation on the injured vascular wall, contributing to microvascular plugging and inflammation in brain ischemia-reperfusion. A disintegrin and metalloproteinase with thrombospondin type-1 motifs 13 (ADAMTS13) cleaves the VWF multimer strand and reduces its prothrombotic and proinflammatory functions. Although ADAMTS13 deficiency is known to amplify post-ischemic cerebral hypoperfusion, there is no report available on the effect of ADAMTS13 on inflammation after brain ischemia. We investigated if ADAMTS13 deficiency intensifies the increase of extracellular HMGB1, a hallmark of post-stroke inflammation, and exacerbates brain injury after ischemia-reperfusion. ADAMTS13 gene knockout (KO) and wild-type (WT) mice were subjected to 30-min middle cerebral artery occlusion (MCAO) and 23.5-h reperfusion under continuous monitoring of regional cerebral blood flow (rCBF). The infarct volume, plasma high-mobility group box1 (HMGB1) level, and immunoreactivity of the ischemic cerebral cortical tissue (double immunofluorescent labeling) against HMGB1/NeuN (neuron-specific nuclear protein) or HMGB1/MPO (myeloperoxidase) were estimated 24h after MCAO. ADAMTS13KO mice had larger brain infarcts compared with WT 24h after MCAO ($p<0.05$). The rCBF during reperfusion decreased more in ADAMTS13KO mice. The plasma HMGB1 increased more in ADAMTS13KO mice than in WT after ischemia-reperfusion ($p<0.05$). Brain ischemia induced more prominent activation of inflammatory cells co-expressing HMGB1 and MPO and more marked neuronal death in the cortical ischemic penumbra of ADAMTS13KO mice. ADAMTS13 deficiency may enhance systemic and brain inflammation associated with HMGB1 neurotoxicity, and aggravate brain damage in mice after brief focal ischemia. We hypothesize that ADAMTS13 protects brain from ischemia-reperfusion injury by regulating VWF-dependent inflammation as well as microvascular plugging

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ORIGINAL ARTICLE

ADAMTS13 gene deletion enhances plasma high-mobility group box1 elevation and neuroinflammation in brain ischemia–reperfusion injury

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Abstract Highly adhesive glycoprotein von Willebrand factor (VWF) multimer induces platelet aggregation and leukocyte tethering or extravasation on the injured vascular wall, contributing to microvascular plugging and inflammation in brain ischemia–reperfusion. A disintegrin and metalloproteinase with thrombospondin type-1 motifs 13 (ADAMTS13) cleaves the VWF multimer strand and reduces its prothrombotic and proinflammatory functions. Although ADAMTS13 deficiency is known to amplify post-ischemic cerebral hypoperfusion, there is no report available on the effect of ADAMTS13 on inflammation after brain ischemia. We investigated if ADAMTS13 deficiency intensifies the increase of extracellular HMGB1, a hallmark of post-stroke inflammation, and exacerbates brain injury after ischemia–reperfusion. ADAMTS13 gene knockout (KO) and wild-type (WT) mice were subjected to 30-min middle cerebral artery occlusion (MCAO) and 23.5-h reperfusion under continuous monitoring of regional cerebral blood flow (rCBF). The infarct volume, plasma high-mobility group box1 (HMGB1) level, and immunoreactivity of the ischemic cerebral cortical tissue (double immunofluorescent labeling) against HMGB1/NeuN (neuron-specific nuclear protein) or HMGB1/MPO (myeloperoxidase) were estimated 24 h after MCAO. ADAMTS 13KO mice had larger brain infarcts compared with WT 24 h after MCAO ($p < 0.05$). The rCBF during reperfusion decreased more in ADAMTS13KO mice. The plasma HMGB1 increased more in ADAMTS13KO mice than in WT after ischemia–reperfusion ($p < 0.05$). Brain ischemia induced more prominent activation of inflammatory cells co-expressing HMGB1 and MPO and more marked neuronal death in the cortical ischemic penumbra of ADAMTS13KO mice. ADAMTS13 deficiency may enhance systemic and brain inflammation associated with HMGB1 neurotoxicity, and aggravate brain damage in mice after brief focal ischemia. We hypothesize that ADAMTS13 protects brain from ischemia–reperfusion injury by regulating VWF-dependent inflammation as well as microvascular plugging.

Keywords Brain ischemia–reperfusion · High-mobility group box1 · ADAMTS13 · Inflammation · Von Willebrand factor · Thrombotic thrombocytopenic purpura

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Introduction

The post-ischemic inflammation incites stroke evolution [15, 16, 19–21, 32]. An ischemic insult triggers leukocytes infiltration and astrocyte and microglia activations in the affected brain, leading to the increase of the high-mobility group box1 (HMGB1) in the plasma and brain of the stroke model [19, 21]. The HMGB1 is a potent proinflammatory cytokine secreted by blood-immune [23, 29, 45] and brain-glial cells [35]. This extracellular HMGB1 further activates monocytes/macrophages [3, 34], neutrophils [1, 34], microvascular endothelial cells [12], astrocytes [36] and microglia [24], amplifies the systemic and brain inflammation, and extends the ischemic brain damage into the penumbra [19, 21, 24, 27, 33, 38].

Within the ischemic brain vasculature after middle cerebral artery occlusion (MCAO), circulating platelets [2, 28] and leukocytes [9, 28, 31] are activated, inducing microvascular obstructions and inflammation. In the initial activations of platelet and leukocyte on ischemic endothelium, a large multimeric adhesive glycoprotein von Willebrand factor (VWF) plays a central role. The VWF multimer tethers platelets on the vascular endothelial surface, leading to platelet activation [40]. This platelet-decorated VWF multimer string bound to endothelium supports leukocytes tethering, rolling and transmigration on stimulated vascular endothelial cells, and links thrombosis to inflammation [5, 7, 37]. The platelet binding affinity of VWF increases with increasing length of the VWF multimer strand and with high fluid shear stress [40, 44]. Accordingly, the longest multimer termed ultra-large VWF (ULVWF; secreted by vascular endothelium upon stimulation) exerts its maximum prothrombotic and pro-inflammatory functions in the microvasculture or stenotic vessels under high shear stress condition [30, 40, 41, 43].

A disintegrin and metalloproteinase with thrombospondin type-1 motifs 13 (ADAMTS13) inhibits these VWF functions by cleaving the Tyr1605–Met1606 bond in the A2 domain of the VWF [13, 41]. Physiologically, circulating ADAMTS13 cleaves the ULVWF secreted from endothelial cells, releasing tethered platelets and VWF fragments [11]. In a setting of on-going thrombus formation, the high shear stress induced at the stenotic vasculature stretches plasma-derived VWF multimers (smaller than ULVWF) on the thrombus surface. The extended VWF multimers involved in the platelet thrombosis are consequently cleaved by ADAMTS13 [41]. Notably, by decreasing the interaction between the ULVWF–platelet strands and leukocytes, ADAMTS13 reduces leukocytes adhesion and extravasation on the stimulated vascular wall and down-regulates tissue inflammation [5, 7, 37]. ADAMTS13 deficiency in humans increases the circulating ULVWF resulting in thrombotic thrombocytopenic purpura (TTP) [17, 41]. The TTP manifests fever and neurological deficits associated with VWF–platelet microthrombus formation in the brain. This implies that ADAMTS13 plays a role in inflammation after brain ischemia in TTP patients.

Early studies have shown that ADAMTS13 deficiency aggravates ischemic brain damage in experimental stroke models [14, 48]. We revealed that in the ADAMTS13-deficient mice after a brief focal ischemia the post-ischemic hypoperfusion was significantly amplified partly because of enhanced microvascular plugging by VWF–platelet–leukocyte complex [14]. However, it still remains unclear if an enhanced inflammatory reaction is involved in the deterioration of ischemic brain injury under ADAMTS13 deficiency. Here, we investigated whether ADAMTS13 gene deletion intensifies the increase of extracellular HMGB1, a hallmark of post-stroke inflammation, and exacerbates the brain damage after ischemia–reperfusion.

Materials and methods

Animals

The effect of ADAMTS13 gene deletion on inflammation after brain ischemia was investigated using male ADAMTS13KO and littermate WT mice in an SV129 genetic background, originally generated as a TTP model by our study group [4]. Studies using KO ($n = 31$) and WT ($n = 31$) mice (8–10 weeks of age, 20–23 g of body weight) were approved by the institutional ethics committee. The genotype of each animal was kept unspecified until all experiment's completion.

Middle cerebral artery occlusion

Thirty-minute MCAO by thread insertion from the common carotid artery was induced in KO ($n = 21$) and WT ($n = 21$) mice as previously described [14, 19–21]. Mice were anesthetized with 2% halothane for induction and maintained on 1% halothane in 70% N_2O and 30% O_2 by face mask. Body temperature was maintained at 36.5–37.0°C during surgery. Successful left MCAO was confirmed according to the following criteria: (1) rCBF in the left cerebral cortex at the thread insertion less than 20% of the pre-MCAO rCBF, and (2) consistent presence of significant ischemic neurological symptoms of the left cerebral hemisphere, characterized by right paresis and right circling behavior, during 30-min MCAO. The MCAO surgery was performed within 7 min without bleeding. The anesthesia was discontinued during 30-min MCAO. There were no statistically significant differences in body temperature between the two groups immediately before or after the thread insertion, or 10, 20, and 30 min after

MCAO. The thread was removed under re-anesthesia after 30-min MCAO. Sham surgery in KO ($n = 10$) and WT ($n = 10$) involved temporary insertion (1 s) of the thread into the left common carotid artery without MCAO. There were no statistical differences in prothrombin time or survival rate between KO and WT mice at 24 h after MCAO (Table 1).

Regional cerebral blood flow

The rCBF was measured by laser Doppler flowmetry (LDF) (ALF21, Advance Co., Tokyo, Japan) as previously described [14, 20]. The LDF probe was placed through a guide cannula into the left cerebral cortex stereotaxically (0.22 mm posterior and 2.5 mm lateral from bregma; 1.5 mm depth from the skull surface) on a stereotaxic instrument (Narishige Scientific Instrument Lab: SR-5 M, Tokyo) under anesthesia (pentobarbital 50 mg/kg, i.p.) 24 h before MCAO or sham surgery. The rCBF was monitored in all animals continuously from 30 min before MCAO until immediately after reperfusion. In randomly selected animals, the rCBF was repeatedly recorded over time within 24 h after MCAO. The rCBF during occlusion and reperfusion was expressed as percentages of the preMCAO LDF baseline value.

Infarct volume and neurological deficit

The brains were sectioned coronally (four 2-mm thick slices) according to a mouse brain matrix 24 h after MCAO (KO, $n = 14$ and WT, $n = 15$) or sham operation (KO, $n = 5$ and WT, $n = 5$). The infarct area was measured in each slice stained with 2,3,5 triphenyltetrazolium chloride with an image analysis system (NIH Image, version 1.63), and the infarct volume was calculated [14, 19–21]. Neurological deficit score ranging from 0 (normal motor function) to 5 (no spontaneous motor activity) was measured at 24 h after MCAO [14, 19, 21] (Table 1).

Plasma HMGB1 measurement

The plasma HMGB1 protein was evaluated by western blot 24 h after MCAO. Plasma samples were fractionated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and HMGB1 levels were determined by immunoblotting with respect to a standard curve, with recombinant HMGB1 as a reference (Sigma-Aldrich, Tokyo) [19, 21]. A blood sample (500 μL) was taken from each experimental animal via inferior vena cava 24 h after MCAO (KO, $n = 11$ and WT, $n = 12$) or sham surgery (KO, $n = 10$ and WT, $n = 10$). The sample was centrifuged (3,000 rpm at 4°C for 10 min), and the supernatant (200 μL) was further centrifuged (15,000 rpm at 4°C for 20 min). SDS sample buffer [125 mmol/L Tris (pH 6.8), 2% SDS, 20% glycerol, 0.0001% bromophenol blue, and 10% β-mercaptoethanol] (100 μL) was added to the plasma extract solution (100 μL), and the resultant sample was heated at 95°C for 5 min. Protein (15 μg) was separated by SDS–polyacrylamide gel electrophoresis (20% gel). Blotting was performed at 2 mA/cm^2 by semi-dry type blotting (Bio-Rad, Tokyo, Japan). The blots were blocked with 5% non-fat dry milk in Tris-buffered saline in 0.1% Tween 20 (TBS-T) at 4°C and incubated with goat polyclonal anti-HMGB1 primary antibody (1:200) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in TBS-T, followed by bovine anti-goat IgG (heavy chain and light chain [H + L]) alkaline phosphatase conjugate (1:1000) in TBS-T. The blots were visualized with the use of alkaline phosphatase color reagents. The signal intensity of the blots was measured with an image analysis system (NIH Image, version 1.63).

Double immunohistochemical staining for HMGB1/NeuN and HMGB1/MPO

Double immunofluorescent labeling for HMGB1 with NeuN or MPO on paraffin-embedded 5-μm coronal sections

Table 1 Effect of ADAMTS13 gene deletion on brain ischemia in mice after 30-min MCAO and 23.5-h reperfusion

	WT		KO
Brain infarct volume (mm^3)	12.0 ± 2.0 ($n = 15$)	$p < 0.05$[a]	28.5 ± 5.8 ($n = 14$)
Neurological deficit score	1.3 ± 0.2 ($n = 15$)	$p < 0.05$[a]	1.9 ± 0.2 ($n = 14$)
Prothrombin time (s)	12.1 ± 0.8 ($n = 7$)	ns	11.6 ± 0.9 ($n = 6$)
Survival rate (%)	95.2 ($n = 20/21$)	ns	90.4 ($n = 19/21$)

The values are expressed as the mean ± SEM. Neurological deficit score, score 0; normal motor function, 1; flexion of torso and of contralateral forelimb upon lifting of the animal by the tail, 2; circling to the ipsilateral side but normal posture at rest, 3; circling to the ipsilateral side, 4; rolling to the ipsilateral side, 5; leaning to the ipsilateral side at rest (no spontaneous motor activity)

ns Statistically not significant

[a] Student *t* test

of the mouse brain was analyzed by fluorescence microscopy (Nikon, AZ-FL, Tokyo, Japan). At 24 h after MCAO (KO, $n = 5$; WT, $n = 5$) or sham surgery (KO, $n = 5$; WT, $n = 5$), mice were humanely perfused transcardially with saline and 4% paraformaldehyde. The brains were removed of fat and water using an autodegreasing unit (RH-12; Sakura Seiko Co, Tokyo) and embedded in paraffin. Subsequently, 5-μm sections were mounted on slides and dried at 37°C for 1 day. After deparaffinization and rehydration, the sections were incubated with primary antibodies of biotinylated anti-mouse NeuN (1:200; Chemicon International, Temecula, CA, USA) or rabbit polyclonal anti-MPO (1:200; DAKO Inc., Carpinteria, CA, USA) and of goat polyclonal anti-HMGB1 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. Sections were then incubated with donkey anti-goat IgG-FITC secondary antibody (1:200; Santa Cruz Biotechnology) for 1 h, and thereafter with goat anti-rabbit IgG-Texas red secondary antibody (1:200; Santa Cruz Biotechnology) or Ultra avidine Texas Red (1:200; Leinco Technologies) for 1 h. The sections were imaged and analyzed. The histological findings were evaluated by neuropathologists until a consensus was obtained. The fluorescence intensity (for cells positive to NeuN, MPO, or HMGB1) in five randomly selected areas (150 μm × 200 μm for each) from the region of interest in the ischemic cerebral cortex (as indicated in Figs. 2, 3) was evaluated with an image analysis system (NIH Image, version 1.63) with the corresponding non-ischemic contralateral regions as a control, and the relative fluorescence intensity was calculated. In ischemic stroke, the necrotic core is surrounded by a zone of reactive/inflammatory cytosis which can extend the initial insult into the penumbra with delayed cell death. Based on this concept, the region of interest in the cortical penumbra for the fluorescence evaluation was decided as indicated.

Statistical analysis

Data are presented as mean ± standard error of mean (SEM). For multiple pairwise comparisons in parametric analysis, two-way analysis of variance (ANOVA) followed by Tukey–Kramer's test was performed. When only two groups were compared, Student's *t* test was used. Probability values of <0.05 were considered statistically significant.

Results

Brain infarction

The ADAMTS13 gene knockout (ADAMTS13KO) mice group had a significantly larger volume of brain infarction compared with the wild-type (WT) following 23.5-h reperfusion after 30-min MCAO (Student's *t* test) (Table 1). No ischemic brain damage was observed in either KO or WT mice after sham operation.

Neurological deficits

ADAMTS13KO mice had more severe neurological deficits than the WT (Student's *t* test) 24 h after MCAO (Table 1).

Regional cerebral blood flow

The rCBF showed no statistical differences between the two groups during MCAO or immediately after reperfusion. However, the rCBF in ADAMTS13KO mice progressively decreased significantly more markedly compared to WT during the first 30-min reperfusion (Tukey–Kramer's test). 24 h after MCAO, the rCBF in KO mice remained significantly lower compared to WT (Student's *t* test) (Table 2).

ADAMTS13 gene deletion enhances post-ischemic increase of plasma HMGB1

A 30-min MCAO and 23.5-h reperfusion significantly increased the plasma HMGB1 level both in ADAMTS13KO and WT mice as compared to sham operation (Fig. 1). However, this increase of plasma HMGB1 after MCAO was more markedly enhanced in ADAMTS13KO mice than in WT (Tukey–Kramer's test).

ADAMTS13 gene deletion intensifies post-ischemic brain inflammation and neuronal death

Double immunohistochemical staining for HMGB1/NeuN and HMGB1/MPO

The qualitative analysis of double immunofluorescent labeling for HMGB1 with NeuN (neuron-specific nuclear protein) or MPO (myeloperoxidase) on the cortical tissue 24 h after MCAO or sham operation showed that (1) the number of neurons immunoreactive to NeuN in the ischemic penumbra decreased more in ADAMTS13KO mice compared to WT (Fig. 2), (2) HMGB1 immunoreactivity disappeared in the ischemic neuronal nuclei in both KO and WT mice, suggestive of a translocation of HMGB1 from neuronal nucleus either to neuronal cytoplasm or to extracellular space (Fig. 2), and (3) cells with co-expression of MPO (a marker for neutrophils, macrophages and/or microglia) and HMGB1 appeared more prominently in the ischemic penumbra in ADAMTS13KO mice than in WT (Fig. 3).

Table 2 Effect of ADAMTS13 gene deletion on regional cerebral blood flow (rCBF) in mice of 30-min MCAO model

	WT ($n = 6$)		KO ($n = 7$)
Time (min)			
Baseline	100.0 ± 0	ns	100.0 ± 0
0	13.6 ± 4.6	ns	13.2 ± 2.0
10	17.9 ± 3.4	ns	22.4 ± 6.3
20	17.7 ± 5.2	ns	19.3 ± 5.7
30	16.8 ± 4.7	ns	18.6 ± 3.5
Reperfusion	114.3 ± 19.1	ns	88.1 ± 8.1
40	90.5 ± 12.7	$p < 0.05$[a]	48.4 ± 8.4
50	93.8 ± 11.3	$p < 0.01$[a]	35.4 ± 7.7
60	83.2 ± 6.8	$p < 0.01$[a]	28.1 ± 4.7
Time (hour)			
24	72.9 ± 13.9	$p < 0.05$[b]	37.2 ± 5.9

rCBF values are expressed as the mean ± SEM (% of baseline)

[a] Turkey-Kramer's test after two-way repeated measures ANOVA [$F(8,98) = 5.841$, $p < 0.0001$)]

[b] Student's t test

Relative fluorescent intensity of NeuN, MPO and HMGB1

The relative fluorescent intensity (%) in the ischemic cerebral cortex 24 h after MCAO was significantly decreased for NeuN in ADAMTS13KO mice compared to WT [57.5 ± 8.7 in KO vs. 93.2 ± 5.5 in WT ($p < 0.05$, student's t test)], and increased for both MPO and HMGB1 more in ADAMTS13KO mice than in WT [KO vs. WT: 557.7 ± 139.8 vs. 310.4 ± 131.7 for MPO, and 159.7 ± 46.5 vs. 70.2 ± 35.1 for HMGB1 (although not statistically significant)].

Discussion

The (UL)VWF, the substrate of ADAMTS13, recruits platelets and leukocytes onto the injured vascular endothelium, and mediates microvascular plugging and enhances the tissue inflammation [5, 7, 30, 37, 40, 41, 43, 44]. ADAMTS13 inhibits these prothrombotic and proinflammatory functions of (UL)VWF [7, 13, 41]. Our current study implies that ADAMTS13 gene deletion amplifies systemic and brain inflammatory responses against brain ischemia–reperfusion enhancing a potent cytokine HMGB1 neurotoxicity, leads to progressive decline of post-ischemic cerebral blood reflow, and exacerbates ischemic brain injury. ADAMTS13 may play a neuroprotective role against inflammation in ischemic stroke.

ADAMTS13 deficiency may promote inflammation by activating platelets, leukocytes, and vascular endothelium after brain ischemia–reperfusion. Responding to ischemia–

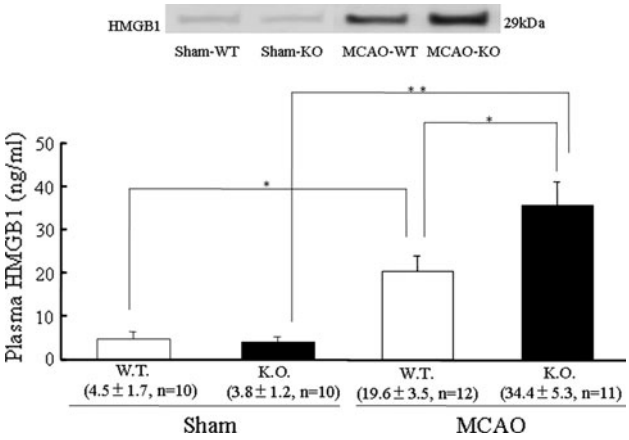

Fig. 1 Effect of ADAMTS13 gene deletion on plasma HMGB1 in mice after 30-min MCAO. The plasma HMGB1 protein was evaluated by western blot. Transient focal ischemia of 30-min MCAO followed by 23.5-h reperfusion significantly increased the plasma HMGB1 level both in ADAMTS13KO and WT mice when compared to sham operation [plasma HMGB1 (ng/ml): MCAO-KO; 34.4 ± 5.3 vs. sham-KO; 3.8 ± 1.2, $p < 0.01$, and MCAO-WT; 19.6 ± 3.5 vs. sham-WT; 4.5 ± 1.7, $p < 0.05$, Tukey–Kramer's test after two-way ANOVA ($F(1,40) = 38.401$, $p < 0.01$)]. This increase of plasma HMGB1 at 24 h after MCAO was more markedly enhanced in ADAMTS13KO mice than in WT [MCAO-KO; 34.4 ± 5.3 vs. MCAO-WT; 19.6 ± 3.5, $p < 0.05$, Tukey–Kramer's test after two-way ANOVA ($F(1,40) = 4.296$, $p < 0.05$)]. Sham-WT $n = 10$, Sham-KO $n = 10$, MCAO-WT $n = 12$, MCAO-KO $n = 11$. Values are expressed as the mean ± SEM. *$p < 0.05$, **$p < 0.01$, Tukey–Kramer's test after two-way ANOVA

reperfusion, the stimulated vascular endothelial cells secrete ULVWF [44]. Binding of VWF to the platelet membrane glycoprotein initiates a signaling cascade that causes platelet activation [25, 46]. The activated platelets release multiple proinflammatory factors, mitogenic mediators, metalloproteinases, and reactive oxygen species, and stimulate the leukocytes and endothelium to incite inflammatory reactions [8, 10]. Further, the platelet–(UL)VWF string directly supports the leukocyte transmigration into the inflammatory tissue [5, 7, 37]. Thus, the VWF-cleaving protease ADAMTS13 plays a role as an anti-inflammatory factor, and therefore its deficiency can exaggerate the post-ischemic inflammation.

In ADAMTS13KO mice after a cerebral ischemia, the plasma HMGB1 increased more and the HMGB1-expressing immune cells appeared more prominent in the cortical penumbra than in WT. The increased extracellular HMGB1 may contribute to secondary ischemic brain damage in ADAMTS13KO mice. The HMGB1, a DNA-binding protein, is a central proinflammatory cytokine [29]. Upon inflammatory signals, the chromosomal HMGB1 relocates into cytoplasmic secretory lysosomes and is secreted into the immunological synapse [29] or into the extracellular space by monocytes/macrophages [45], neutrophils [23], mature dendritic cells [29], natural killer cells

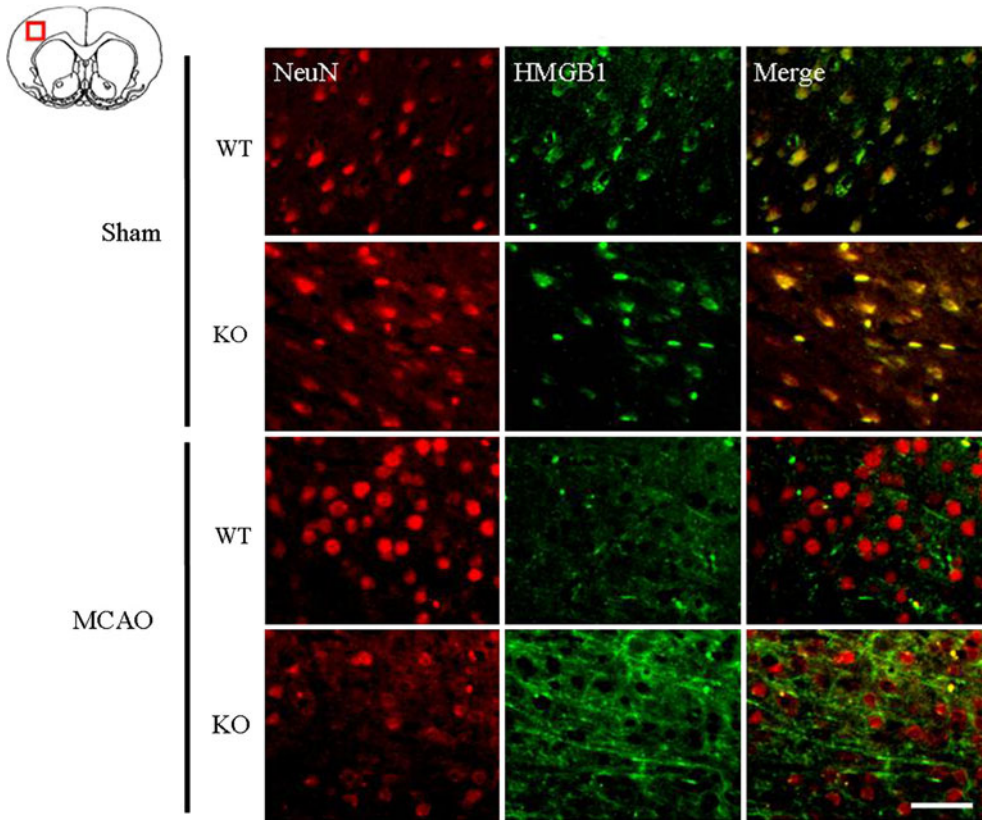

Fig. 2 Effect of ADAMTS13 gene deletion on NeuN positive cells expressing HMGB1 in mice brain after 30-min MCAO. Qualitative analysis of double immunofluorescent labeling for HMGB1 with NeuN on the brain tissue 24 h after MCAO showed that the number of neurons immunoreactive to NeuN in the ischemic cortical penumbra decreased more in ADAMTS13KO mice than WT. The HMGB1 immunoreactivity disappeared in the ischemic neuronal nuclei in both KO and WT mice, suggesting that neuronal-nuclear HMGB1 translocated into either the neuronal cytoplasm or the extracellular space. In addition, the HMGB1 immunoreactivity seemed to increase in the ischemic cortical tissue in the ADAMTS13KO mice, indicating a possibility that non-neuronal HMGB1 positive cells were activated in the KO mice compared to the WT ($n = 5$ in each group). *Scale bar* 50 μm. NeuN positive cells *red*, HMGB1 positive cells *green*, merge *yellow*

[29], and glia [35]. HMGB1 also leaks from necrotic cells [42] and ischemic neurons [38]. The extracellular HMGB1 binds to its receptors, RAGE (receptor for advanced glycation end products) [22], Toll-like receptor 2 (TLR2), and TLR4 [34], recapitulates the intracellular signaling cascades initiated by early proinflammatory stresses, and thus propagates continuous proinflammatory responses [3, 22, 29, 34, 42]. Naturally, high blood HMGB1 correlates with the severity of systemic inflammation [29, 45].

After brain ischemia, extracellular HMGB1 increases rapidly in the blood and central nervous system, and induces neuroinflammation [24, 38]. HMGB-1 early released from the striatal ischemic core can bind to RAGE that is robustly expressed in the peri-infarct region, and extend the ischemic brain injury [38]. HMGB1-RAGE signaling in infiltrating macrophages and activated microglia seemingly mediate neuronal death evolution in the ischemic penumbra [33]. The HMGB1 inhibition in the brain or systemic circulation protects the blood–brain barrier and the brain from ischemia [24, 27, 33, 47]. Inhibition of leukocytes and microglia results in decreased HMGB1 levels in the brain and plasma, reduces apoptosis in the ischemic brain, and improves brain atrophy and neurologic deficits [19, 21]. Therefore, the increased extracellular HMGB1 in the blood and brain of ADAMTS13KO mice as observed in our study can exacerbate ischemic brain injury by intensifying systemic and brain inflammation. Interestingly, the platelet intracellular HMGB1 is exported to the external surface of the plasma membrane upon its activation [39]. Accordingly, the activated platelet may be an additional source of the exceedingly increased plasma HMGB1 in ADAMTS13KO mice after brain ischemia, where enhanced VWF–platelet interactions develop [14]. We suggest that ADAMTS13 deficiency multiplies secondary insults after brain ischemia by up-regulating (UL)VWF-mediating inflammation and enhancing HMGB1 neurotoxicity in the systemic and local environments.

This study suggests a potential therapy with ADAMTS13 for acute ischemic stroke by breaking a vicious circle

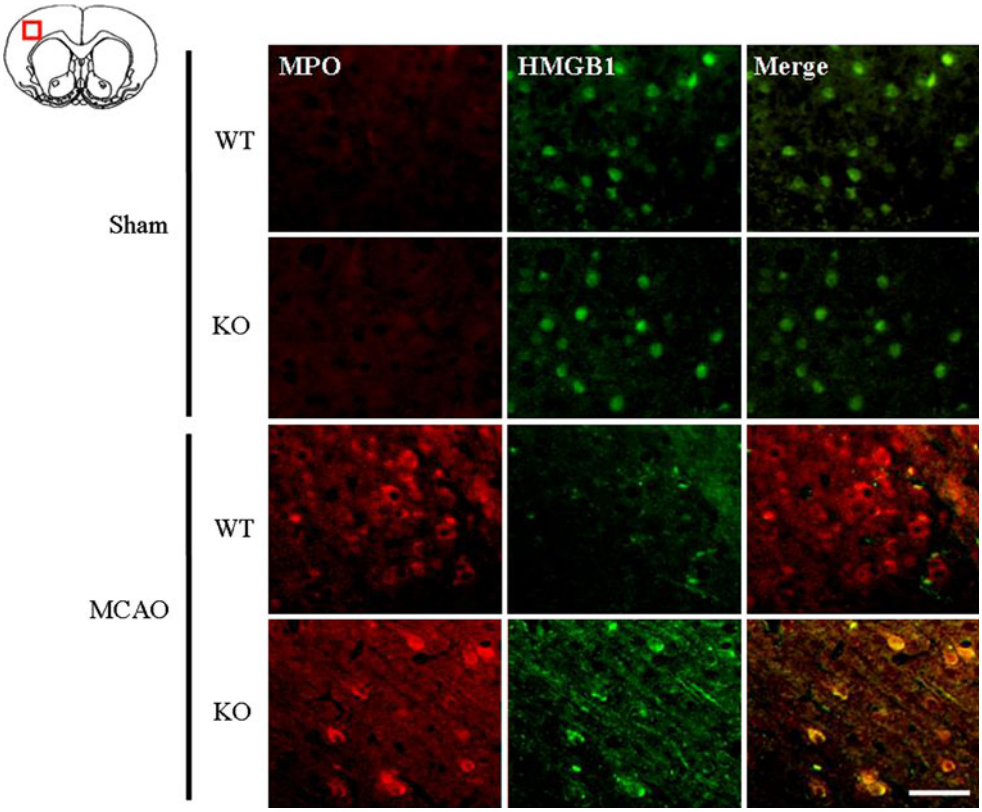

Fig. 3 Effect of ADAMTS13 gene deletion on MPO positive cells expressing HMGB1 in mice brain after 30-min MCAO. Qualitative analysis of double immunofluorescent labeling for HMGB1 with MPO on the brain tissue 24 h after MCAO showed that cells co-expressing MPO and HMGB1 were more prominent in the ischemic cortical penumbra in KO mice than in WT ($n = 5$ in each group). *Scale bar* 50 μm. MPO positive cells *red*, HMGB1 positive cells *green*, merge *yellow*

of thrombosis and inflammation. The (UL)VWF–platelet string interacts with leukocytes, and provokes inflammation [5, 7, 37]. The inflammation induces the endothelial-ULVWF secretion [44]. The proinflammatory cytokines from leukocytes and endothelial cells [such as tumor necrosis factor (TNF)-α and interleukin (IL)-8] stimulate the endothelial ULVWF release and IL-6 protects the ULVWF from cleavage [6]. This would increase the number of ULVWF multimers in plasma sufficiently to aggregate platelets and on vascular endothelial surface to tether platelets and leukocytes onto the endothelium, providing a linkage between thrombosis and inflammation. Of note, HMGB1 stimulates the monocytes/macrophages [3, 34], neutrophils [1, 34] and glial cells [24, 36, 38] to produce TNF-α, IL-1, IL-6 and/or IL-8, and incites the microvascular endothelial cells [12, 38] to express TNF-α, IL-8 and various adhesion molecules. Namely, the increased plasma HMGB1 in ADAMTS13-deficient mice can upregulate ULVWF, and thus reinforce the association between inflammation and thrombosis. ADAMTS13 may prevent stroke evolution by interfering with the crosstalk between thrombosis and inflammation.

Thrombolytic therapy using tissue plasminogen activator (tPA) for acute stroke has limitations in the therapeutic time window and in the drug dosage due to the risk of hemorrhagic transformation [18]. Further, tPA directly exerts neurotoxicity in the ischemic brain [26]. We suggest that a regulation of the interaction between (UL)VWF–platelet and leukocyte using ADAMTS13 may become a novel therapeutic option in acute brain ischemia. ADAMTS13 does not dissolve the VWF–platelet–primary hemostatic thrombus in the absence of pathologically high fluid shear stress. Therefore, ADAMTS13 may be particularly well suited for acute ischemic stroke without increasing hemorrhagic complications. An early experimental study [48] together with our preliminary data (not shown) demonstrated that recombinant human ADAMTS13 administration reduced infarct volume in stroke model in a VWF-dependent manner without producing cerebral hemorrhage.

This study has several limitations. For example, the reduction of cerebral blood reflow in ADAMTS13KO mice after ischemic insult was continuous and higher than that observed in WT. Therefore, even without the amplified inflammation with HMGB1 neurotoxic effects, only the difference in the blood flow recovery might explain the following more deleterious events in the ischemic brain of ADAMTS13-deficient mice compared to WT. The enhanced elevation of the plasma HMGB1 under ADAMTS13 deficiency after brain ischemia might be also explained simply by the more exacerbated brain damage, regardless of the theoretically intensified interactions between the platelet–(UL)VWF strands and the leukocytes without VWF cleaving protease. The future study required to clarify these issues would include chronological data

evaluations in the stroke experiments with permanent ischemic procedure (deleting the reperfusion effect) or with enhancing/inhibiting HMGB1 activities by drugs or genetic manipulations.

Conclusions

A gene deletion of ADAMTS13 renders mice more vulnerable to brain ischemia–reperfusion injury than their wild-type counterparts, when subjected to 30-min MCAO. This preliminary study suggests that ADAMTS13 deficiency may exacerbate systemic and neuronal inflammation after brain ischemia via VWF-dependent pathway, although this remains still hypothetical. Further studies are warranted to better characterize the role of ADAMTS13 in brain ischemia–reperfusion and to provide a novel therapeutic approach for ischemic stroke by regulating VWF-dependent inflammation as well as microvascular plugging.

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